Mutation Research 719 (2011) 35–40

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Genotoxicity assessment of an energetic propellant compound, 3-nitro-1,2,4-triazol-5-one (NTO)

Gunda Reddy[a,*], Jian Song[b], Paul Kirby[b], Emily M. Lent[a], Lee C.B. Crouse[a], Mark S. Johnson[a]

[a] U.S. Army Public Health Command, 5158 Blackhawk Road, Aberdeen Proving Ground, MD, United States
[b] SITEK Research Laboratories, Rockville, MD, United States

ARTICLE INFO

Article history:
Received 10 September 2010
Received in revised form 5 November 2010
Accepted 11 November 2010
Available online 19 November 2010

Keywords:
3-Nitro-1,2,4-triazol-5-one
CAS No. 932-64-9
Genotoxicity
Mutagenicity
Rat micronucleus
Flow cytometry

ABSTRACT

3-Nitro-1,2,4-triazol-5-one (NTO) is an energetic explosive proposed for use in weapon systems, to reduce the sensitivity of warheads. In order to develop toxicity data for safety assessment, we investigated the genotoxicity of NTO, using a battery of genotoxicity tests, which included the Ames test, Chinese Hamster Ovary (CHO) cell chromosome aberration test, L5178Y TK$^{+/-}$ mouse lymphoma mutagenesis test and rat micronucleus test. NTO was not mutagenic in the Ames test or in *Escherichia coli* (WP2*uvrA*). NTO did not induce chromosomal aberrations in CHO cells, with or without metabolic activation. In the L5178Y TK$^{+/-}$ mouse lymphoma mutagenesis test, all of the NTO-treated cultures had mutant frequencies that were similar to the average frequencies of solvent control-treated cultures, indicating a negative result. Confirmatory tests for the three *in vitro* tests also produced negative results. The potential *in vivo* clastogenicity and aneugenicity of NTO was evaluated using the rat peripheral blood micronucleus test. NTO was administered by oral gavage to male and female Sprague–Dawley rats for 14 days at doses up to 2 g/kg/day. Flow cytometric analysis of peripheral blood demonstrated no significant induction of micronucleated reticulocytes relative to the vehicle control (PEG-200). These studies reveal that NTO was not genotoxic in either *in vitro* or *in vivo* tests and suggest a low risk of genetic hazards associated with exposure.

1. Introduction

The energetic explosive 3-nitro-1,2,4-triazol-5-one (NTO) was first prepared in 1905 by nitration of 1,2,4-triazole-5-one (TO) [1]. There was renewed interest in learning more about the chemistry of NTO in the late 1960s. The first report on the explosive nature of NTO was published by Lee and Coburn in 1985 [2]. In the past decade, the military services have been evaluating compounds for explosive formulations to replace highly sensitive explosives, such as 1,3,5-trinitro-1,3,5-triazine (RDX) and 2,4,6-trinitrotoluene (TNT). NTO is reported to be less sensitive and more stable than RDX and TNT [3–5]. By definition, insensitive munitions are those "which reliably fulfill their performance, readiness and operational requirements on demand, and which minimize the probability of inadvertent initiation and severity of subsequent collateral damage to weapon platforms, logistic systems and personnel when subjected to unplanned stimuli" [6]. NTO is presently used in a number of formulations in weapon systems [7]. The US Army Research Development and Engineering Center (USARDEC) is evaluating explosive formulations containing NTO for use in additional weapon systems.

Development of occupational and environmental exposure standards is limited by the lack of toxicity data for NTO. The acute oral LD_{50} for NTO is reported to be >5 g/kg in rats and mice. In rabbit tests, NTO produced mild skin irritation but was not an eye irritant and did not induce dermal sensitization in guinea pigs [8]. A recently completed oral subchronic toxicity test in rats at doses of 0, 30, 100, 315 and 1000 mg/kg/day showed no compound-related effects on food consumption or body weight. Reduced testicular size was observed in the 315 and 1000 mg/kg/day groups and microscopic changes in testis were observed in all dose groups. The Lowest Observed Adverse Effect Level (LOAEL) was determined to be 30 mg/kg/day in rats, based on microscopic changes in the testis [9]. There are no published studies on the toxicokinetics and metabolism of NTO in animals. However, the fate of NTO was investigated *in vitro* using rat liver microsomes and bacterial systems. Rat liver microsome catalysis of NTO under nitrogen atmosphere produced primarily amine, 5-amino-1,2,4-triazol-3-one, but, in the presence of oxygen, produced a major product, 5-hydroxy-1,2,4-trizol-3-one urazole, and a minor product, an amine [10,11]. Addition of NTO to aqueous medium releases protons and lowers pH. The metabolism of NTO by bacteria is pH-dependent in aqueous systems. The maximum microbial reduction occurred at pH 6 in the presence of sucrose, while a ring cleavage occurred at pH 8 [11]. NTO also showed pH-dependent toxicity to *Ceriodaphnia dubia*. The growth inhibition value (IC 50) was

* Corresponding author. Tel.: +1 410 436 7701; fax: +1 410 436 8258.
E-mail address: gunda.reddy@us.army.mil (G. Reddy).

1383-5718/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.mrgentox.2010.11.004

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
05 NOV 2010		**00-00-2010 to 00-00-2010**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Genotoxicity assessment of an energetic propellant compound, 3-nitro-1,2,4-triazol-5-one (NTO)	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **U.S. Army Public Health Command,5158 Blackhawk Road,Aberdeen Proving Ground,MD,21010- 540**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

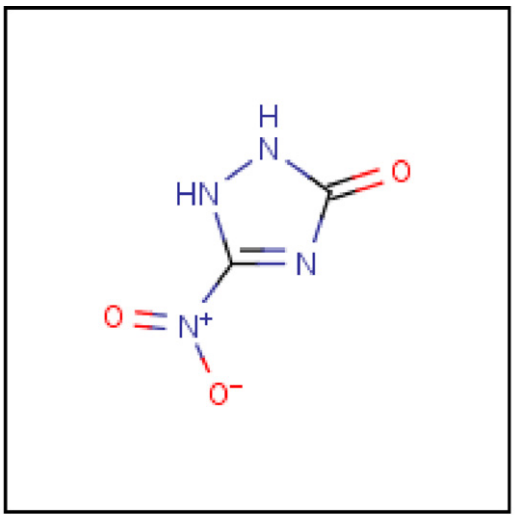
13. SUPPLEMENTARY NOTES

14. ABSTRACT

3-Nitro-1,2,4-triazol-5-one (NTO) is an energetic explosive proposed for use inweaponsystems, to reduce the sensitivity of warheads. In order to develop toxicity data for safety assessment, we investigated the genotoxicity of NTO, using a battery of genotoxicity tests, which included the Ames test, Chinese Hamster Ovary (CHO) cell chromosome aberration test, L5178Y TK+/− mouse lymphoma mutagenesis test and rat micronucleus test. NTO was not mutagenic in the Ames test or in Escherichia coli (WP2uvrA). NTO did not induce chromosomal aberrations in CHO cells, with or without metabolic activation. In the L5178Y TK+/&#8722 mouse lymphoma mutagenesis test, all of the NTO-treated cultures had mutant frequencies that were similar to the average frequencies of solvent control-treated cultures, indicating a negative result. Confirmatory tests for the three in vitro tests also produced negative results. The potential in vivo clastogenicity and aneugenicity of NTO was evaluated using the rat peripheral blood micronucleus test. NTO was administered by oral gavage to male and female Sprague?Dawley rats for 14 days at doses up to 2 g/kg/day. Flow cytometric analysis of peripheral blood demonstrated no significant induction of micronucleated reticulocytes relative to the vehicle control (PEG-200). These studies reveal that NTO was not genotoxic in either in vitro or in vivo tests and suggest a low risk of genetic hazards associated with exposure.

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**Same as Report (SAR)**	**7**	



3-Nitro-1, 2, 4- triazol-5-one (NTO)

Fig. 1. Chemical name and structure.

57 mg/mL after 7 days of exposure when buffered to a neutral pH [12].

There are no published genotoxicity data for NTO. Therefore, we evaluated both *in vitro* and *in vivo* genotoxicity of NTO for health and environmental risk assessment, as part of our comprehensive toxicity and safety evaluation program of new energetic compounds.

2. Materials and methods

2.1. Chemical

Test article: 3-Nitro-1,2,4-triazol-5-one (NTO, 99.6%) pure; CAS# 932-64-9, lot number BAE 07B 305001, was obtained from Ordnance Systems, Inc., Kingsport, TN (Fig. 1). For the Ames test, Aroclor 1254-induced rat hepatic S9 fraction was obtained from Molecular Toxicology, Inc. (Boone, NC); Oxoid Nutrient Broth No. 2 was obtained from Oxoid LTD, Hampshire, England; positive-control substances: 2-nitrofluorene (2-NF), sodium azide (NaAz), 9-aminoacridine (9-AA), methyl methanesulfonate (MMS) and 2-aminoanthracene (2-AA) were obtained from Sigma–Aldrich (St. Louis, MO). For the chromosome aberration test, phenobarbital/β-naphthoflavone-induced rat hepatic S9 fraction was purchased from Molecular Toxicology, Inc. McCoy's 5A cell culture medium, fetal bovine serum, L-glutamine, penicillin and streptomycin were obtained from Gibco-BRL Life Technologies (Grand Island, NY). For the L5178Y $TK^{+/-}$ mouse lymphoma mutagenesis assay, 7 12-dimethylbenz (α) anthracene (DMBA) and trifluorothymidine (TFT) were obtained from Sigma–Aldrich. RPMI medium and horse serum were obtained from Invitrogen, Life Technologies (Chicago, IL). For the rat micronucleus test, ethyl methanesulfonate (EMS) was obtained from Sigma–Aldrich. The *in vitro* tests were performed with NTO dissolved in DMSO. The pH of the test article dosing solutions was adjusted to 6.8–7.0. For the *in vivo* rat micronucleus assay, NTO was suspended in neat polyethylene glycol (PEG)-200 (Mallinckrodt Baker Inc., Phillipsburg, NJ).

These genotoxicity studies were performed in compliance with Good Laboratory Practice and OECD guidelines 471, 473, 474 and 476, adopted July 21, 1997 [13].

2.2. Bacterial reverse mutation test

The *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 were originally obtained from Dr. Bruce N. Ames (University of California, Berkeley, CA). The *Escherichia coli* strain WP2uvrA was obtained from Pharmacia and Upjohn, Inc. (Kalamazoo, MI).

A range-finding toxicity test for NTO was conducted using strains TA100 and WP2*uvrA*, with or without induced rat liver S-9. The plate incorporation method was performed as described previously [14–17]. In the range-finding test, the mean number of revertants in three plates per dose of test article was calculated and compared to the solvent control values. Cytotoxicity was measured by comparing the number of revertants in test article-treated plates to the values for the solvent controls. Based on the results of the range-finding test, NTO was tested at concentrations of 5, 10, 50, 100 and 250 μg/plate for *S. typhimurium* and 100, 250, 500, 750 and 1000 μg/plate for *E. coli* without activation. With activation, the dose levels were 100, 500, 1000, 2500 and 5000 μg/plate for both *S. typhimurium* and *E. coli*.

The criteria for a positive response were twofold increases for TA98 and TA100, and threefold increases for TA1535, TA1537 and WP2*uvrA* in the number of revertants over that of the solvent controls.

2.3. Chromosome aberration test in Chinese Hamster Ovary (CHO) cells

The CHO cell line (clone CHO-W-B1) used in this study was obtained through Environmental Health Research and Testing Laboratories (Lexington, KY) in 1988. These tests were conducted as described previously [17–20]. CHO-W-B1 cells were incubated at 37 °C in an atmosphere of 5% CO_2 for approximately 20–24 h prior to treatment. The metabolic activation mixture consisted of Phenobarbital/β-naphthoflavone induced rat liver homogenate (S-9 fraction) and the cofactor pool. A range finding test was conducted to determine the toxicity of the test article. Relative Cell Growth (RCG) was calculated 18 h after exposure to the test article or controls, and expressed as a percentage ratio (number of cells in the test flask vs. number of cells in the solvent control flask).

The cells were collected, fixed, and dropped on microscope slides. The slides were stained with 5% Giemsa stain and scored for Mitotic Index (MI). A total of 1000 cells were scored from each concentration and the numbers of dividing cells were recorded. The MI and Relative Mitotic Index (RMI) for each concentration were calculated using the following formulae:

$$\mathrm{MI} = \frac{\text{number of dividing cells from 1000 scored cells}}{10}$$

$$\mathrm{RMI} = \frac{\text{test concentration MI}}{\text{solvent control MI}} \times 100$$

Cytotoxicity was evaluated on the basis of the reduction in the RCG and/or RMI. Based on the results of the range-finding test, the definitive chromosome aberration assay was performed using test article concentrations of 5, 50, 100, 500, 1000 and 5000 μg/ml, with or without metabolic activation. Mitomycin-C (MMC) at 0.4 and 0.8 μg/ml and cyclophosphamide (CP) at 7.5 and 12.5 μg/ml were used as the positive controls in the non-activated and activated systems, respectively. Chromosome aberrations were scored from the cells treated with the concentrations of 500, 1000 and 5000 μg/ml with or without activation, based on the cytotoxicity data. The untreated, corresponding solvent control, and one concentration each of the positive controls were also scored. Two hundred metaphases were scored from each concentration and the controls. Statistical analysis was performed using the chi-squared test.

2.4. Mouse lymphoma assay

The L5178Y $TK^{+/-}$ stock cultures were maintained in log phase growth in $RPMI_{10}$ (RPMI 1640 medium, containing 10% horse serum, 0.1% pluronics F68 and 0.011% sodium pyruvate) until used in the study. A pool of cells was prepared from the stock cultures at a concentration of 1.0×10^6 cells/ml in 50% conditioned $RPMI_{10}$ and 50% fresh $RPMI_0$ (RPMI 1640 medium, no serum added, containing 0.1% pluronic F68 and 0.011% sodium pyruvate). A 6.0 ml sample of the cell preparation was dispensed into 50 ml disposable centrifuge tubes resulting in 6×10^6 cells/tube. Each tube was gassed with approximately 5% CO_2 and 95% air, sealed, and placed on a shaker to keep the cells suspended until treatment. This test was performed as described previously [21–24].

In order to determine the NTO concentrations that would produce cytotoxicity, a range finding test was performed with or without activation. After a 4 h exposure period, the cells were pelleted by centrifugation and the test article was removed. The cells were resuspended in $RPMI_{10}$ (30 ml) after two rinses, gassed with 5% CO_2 and 95% air, and incubated at 37 ± 1 °C on a roller drum rotating at 25 ± 2 rpm. The cell population density of each culture was determined 20 and 44 h post-treatment. The Suspension Growth (SG) of each culture was calculated using the following formula:

$$\mathrm{SG} = \frac{\text{Day 1 cell conc.}}{0.2 \times 10^6\ \text{cells/ml}} \times \frac{\text{Day 2 cell conc.}}{\text{Day 1 adjusted cell conc.}}$$

The Relative Suspension Growth (RSG) of each of the test article-treated cultures was determined by calculating its growth relative to the corresponding solvent control cultures' average SG:

$$\mathrm{RSG} = \frac{\text{SG of treated culture}}{\text{average SG of solvent controls}} \times 100$$

MMS and DMBA were positive controls for the non-activated and S-9 activated assays, respectively.

After the expression period in the mutation assays, cultures were selected for cloning, based on their SG. The cultures treated with the five highest concentrations were selected for cloning on soft agar plates, because the test article was found non-toxic at any doses. For each culture selected for cloning, restrictive medium (RM; 100 ml) was dispensed into a flask designated for the addition of the restrictive agent trifluorothymidine (TFT) (allowing the growth of $TK^{-/-}$ cells only) and 100 ml culture medium (CM) was dispensed into a flask designated as a Viable Count (VC) flask. The CM in the VC flask was used to culture an aliquot of cells from each culture cloned to approximate the percentage of viable cells in each culture.

After an 11–12 days incubation period, the number of colonies per TFT and VC plate was determined by counting them with an ARTEK 880 Colony Counter.

The mutant frequency (MF) of each culture that was successfully cloned was determined as a function of viable cells forming colonies. The calculation was per-

formed as follows:

$$\mathrm{MF}/10^6 \text{ viable cells} = \frac{\text{average number of mutants per (RM)/plate}}{\text{average number of colonies in the corresponding VC plates}} \times 200$$

The Relative Cloning Efficiency (RCE) was determined for each culture using the following formula:

$$\mathrm{RCE} = \frac{\text{average VC count of treated culture}}{\text{average VC count of solvent controls}} \times 100$$

The Total Growth (TG) of a culture was calculated as follows:

$$\mathrm{TG} = \frac{\mathrm{RSG} \times \mathrm{RCE}}{100}$$

The TG was calculated for each test article-treated culture that was successfully cloned. All of the calculations were performed using a validated Excel spreadsheet program 2400B1.xls. The Relative Total Growth (RTG) was calculated using the following formula:

$$\mathrm{RTG} = \frac{\text{TG of treated culture}}{\text{average TG of solvent controls}} \times 100$$

A response would be considered negative if:

1. All of the cultures exhibiting a TG of approximately 10% and greater had MF's that were less than twice that of the mean MF of the corresponding solvent control cultures, and
2. There was no evidence of a dose-dependent response.

A response would be considered positive if at least one dose had a MF that was two times or more greater than the average MF of the corresponding solvent control cultures and the response was dose dependent. In evaluating the results, consideration was given to the degree of toxicity exhibited by the culture having the twofold or greater increase in MF and the magnitude of the increase in MF.

2.5. Rat peripheral blood micronucleus assay

The *in vivo* rat blood micronucleus test was conducted using blood samples collected from rats as part of a 14-day subacute study of NTO [9]. Male and female rats were orally dosed daily with NTO at doses of 0, 1000, 1500, and 2000 mg/kg (6 rats/sex/dose). The vehicle control group received neat polyethylene glycol (PEG 200). The positive control group received a single dose of 200 mg/kg of EMS two days prior to study termination. Blood samples were collected from the lateral saphenous vein of rats of NTO, positive and vehicle control treated animals on the 14th day of exposure. Peripheral blood samples were processed for flow cytometric evaluation of micronucleated reticulocytes (MN-RET) as described previously [25].

The samples for micronucleus assay evaluations were prepared using the MicroFlow Plus Kit® (Litron Laboratories) following the manufacturer's instructions [26]. Briefly, peripheral blood (approximately 120 μl) was collected from the saphenous vein by puncturing the vein with an 18 gauge needle and collecting the blood using a pipette with a tip that was pre-coated in anti-coagulant. The blood was placed in a micro-centrifuge tube containing anticoagulant (0.35 ml) and stored at 4–6 °C prior to fixing. The blood/anticoagulant was fixed by rapidly pipetting 180 μl blood/anticoagulant into centrifuge tubes containing 2 ml methanol at −80 °C, vortexing briefly, and returning to −75 to −85 °C for storage until analysis. On the day of analysis by flow cytometry, the fixative was removed from the blood samples by adding ice-cold Buffer C (12 ml) and centrifuging at 400 × *g* for 5 min at 4 °C. Samples were then treated with RNase A and surface marker antibodies conjugated with fluorescein isothiocyanate (FITC) and phycoerythrin (PE). For each sample, an aliquot of blood (20 μl) was added to a flow cytometry tube containing labeling solution (RNase A, anti-CD71-FITC, and anti-platelet-PE; 80 μl). The tubes were protected from the light, incubated 30 min at 2–6 °C, tapped to re-suspend the cells, incubated 15 min at approximately 37 °C, and then 15 min at room temperature. Blood samples were then re-suspended in a DNA staining solution (propidium iodide, PI) immediately prior to analysis, to dye the micronuclei. The population of interest, micronucleated recticulocytes (MN-RET), was identified as those cells having high levels of both CD71-FITC and PI-associated fluorescence. Prior to analysis of samples, the flow cytometer (Coulter Epics XL/MCL; Beckman Coulter, Miami, FL) was set up and calibrated using the standards provided. Anti-CD71, anti-platelet–PE and PI fluorescence signals were detected in the FL1, FL2 and FL3 channels, respectively. 20,000 MN-RETs were analyzed per sample. The data collected from the micronucleus assay were expressed as the percentage of reticulocytes with micronuclei (%MN-RET). The percentage of total erythrocytes that were reticulocytes (%RET) was calculated for each sample as an indication of cytotoxicity.

A one-way analysis of variance (ANOVA) was used to test for significant differences in %MN-RET for female and male rats separately. The Tukey multiple comparison test was used to evaluate the differences between dose groups. The results were considered to be statistically significant at $p < 0.05$. SPSS® version 16.0 (SPSS Inc., Chicago, IL) was used for all analyses.

3. Results

3.1. Bacterial reverse mutation tests

The results of the range-finding test for strain TA100 indicated that NTO was toxic at 500 μg/plate and above, without activation, and 5 mg/plate, with activation, in regard to relative cloning efficiency. The results of the range finding test for WP2*uvrA* indicated NTO was toxic at 1 mg/plate and above without activation. With activation, the revertants were significantly decreased only at 5 mg/plate.

Based on the results of the range finding test, NTO was tested at concentrations of 5, 10, 50, 100 and 250 μg/plate for *S. typhimurium* and 100, 250, 500, 750 and 1000 μg/plate for *E. coli* without activation. With S9 incubation, the dose levels were 100, 500, 1000, 2500 and 5000 μg/plate for both *S. typhimurium* and *E. coli*. The results, with or without metabolic activation, were negative for all strains. The background lawns were normal and the solvent and positive controls fulfilled the requirements of a valid test. The results of the mutation assay indicate that NTO did not induce significant increases in the frequency of revertants for all tester strains in the presence and absence of induced rat liver S-9 plus cofactors when compared to the solvent controls (Table 1). The

Table 1
Summary data from Ames mutagenicity assay for NTO.

Activation	Treatment (μg/plate)	Mean revertants per plate with standard deviation				
		TA 98	TA 100	TA 1535	TA 1537	WP2*uvr*A
−S9	Solvent (water)	29 ± 7	105 ± 2	12 ± 3	13 ± 1	25 ± 2
	5	36 ± 4	115 ± 14	7 ± 1	16 ± 4	33 ± 8
	10	32 ± 5	122 ± 13	12 ± 3	15 ± 1	20 ± 3
	50	27 ± 2	116 ± 5	12 ± 2	10 ± 2	37 ± 2
	100	32 ± 4	112 ± 7	12 ± 7	14 ± 5	22 ± 3
	250	36 ± 10	116 ± 10	12 ± 2	7 ± 2	30 ± 9
	Positive[a] control	633 ± 47[b]	306 ± 35[b]	234 ± 6[b]	57 ± 16[*]	558 ± 7[b]
+S9	Solvent (water)	52 ± 10	135 ± 10	33 ± 4	22 ± 3	35 ± 6
	100	58 ± 5	140 ± 11	32 ± 1	30 ± 7	26 ± 5
	500	50 ± 11	149 ± 16	29 ± 1	25 ± 3	25 ± 2
	1000	47 ± 8	160 ± 5	36 ± 2	28 ± 8	22 ± 8
	2500	36 ± 3	168 ± 2	43 ± 7	24 ± 2	28 ± 6
	5000	31 ± 3	159 ± 68	30 ± 4	17 ± 3	21 ± 3
	Positive[c] control	1237 ± 162[b]	583 ± 93[*]	161 ± 12[b]	402 ± 27[*]	235 ± 54[b]

[a] TA98 2-nitrofluorene 5.0 μg/plate; TA100 sodium azide 1.0 μg/plate; TA 1535 sodium azide 1.0 μg/plate; TA 1537 9-aminoacridine 75.0 μg/plate; WP2*uvrA* methyl methanesulfonate 4000 μg/plate.
[b] Mutagenic.
[c] TA98, TA100, TA 1535, TA 1537 2-aminoanthracene 2.5 μg/plate; WP2*uvrA* 2-aminoanthracene 20.0 μg/plate.

Table 2
Summary data from CHO chromosome aberration assay for NTO.

Treatment	Concentration (μg/ml)	S9	Cell survival %	Number of cells analyzed	Number of structural aberrations						% cells with aberrations	Number of polyploidy cells (%)
					gap	ctb	cte	csb	cse	oth		
Solvent		–		200	0	2	0	0	0	0	1.0	1.5
NTO	500	–		200	0	2	0	0	0	0	1.0	1.5
	1000	–		200	0	0	0	0	0	0	0	3.0
	5000	–		200	0	0	0	0	0	0	0	1.5
MMC	0.8	+		200	0	30	74	16	2	7	44.5*	3.0
Solvent		+			0	2	0	0	0	0	1.0	5.0
NTO	500	+		200	0	0	0	0	0	0	0	5.0
	1000	+		200	0	1	0	0	0	0	0.5	2.5
	5000	+		200	0	1	0	0	0	0	0.5	5.5
CP	7.5	+		200	0	24	45	9	6	5	34.5*	5.5

Note: ctb, chromatid break; cte, chromatid exchange; csb, chromosome break; cse, chromosome exchange; oth, others; MMC, mitomycin; CP, cyclophosphamide.
* Significantly greater than the corresponding solvent control ($p < 0.05$).

negative result was confirmed by a repeat mutation assay (data not shown).

3.2. CHO cell chromosome aberration assay

The results of the range finding test indicated that NTO was not cytotoxic to CHO cells up to 5 mg/ml, without or with metabolic activation (data not shown). The chromosome aberration assay was performed using NTO at concentrations of 5, 50, 100, 500, 1000 and 5000 μg/ml both without and with metabolic activation. Concurrent solvent and positive controls were also included.

Chromosome aberrations were scored from the cells treated with the concentrations of 500, 1000 and 5000 μg/ml, without or with activation. The untreated controls, the solvent control and one concentration each of the positive controls (MMC at 0.8 μg/ml and CP at 7.5 μg/ml) were also scored. The results from the assay were negative with or without activation (Table 2). Both the solvent and positive controls in the definitive chromosome aberration assay fulfilled the requirements of a valid test. The results from the confirmatory assay were also negative without activation (data not shown).

3.3. Mouse lymphoma assay

The objective of the mouse lymphoma assay was to evaluate the test chemical for its ability to induce forward mutation in L5178Y $TK^{+/-}$ mouse lymphoma cells. Mutant cells are assessed by colony growth in the presence of TFT. The range-finding cytotoxicity test

Table 3
Summary of mouse lymphoma mutation assay with NTO.

Test condition	Concentration (μg/ml)	S9	Total mutant colonies	Total viable colonies	Relative total growth (%)	Mutant frequency ($\times 10^{-6}$)
DMSO A		–	91 ± 9	167 ± 26		109
DMSO B		–	64 ± 4	174 ± 15		74
NTO	250 A	–	138 ± 1	232 ± 28	72	119
	250 B	–	102 ± 9	188 ± 12	122	109
	500 A	–	88 ± 6	225 ± 32	99	78
	500 B	–	124 ± 6	247 ± 24	120	100
	1000 A	–	125 ± 6	274 ± 26	118	91
	1000 B	–	110 ± 21	250 ± 27	132	88
	2500 A	–	107 ± 21	235 ± 11	109	91
	2500 B	–	136 ± 9	309 ± 28	106	88
	5000 A	–	103 ± 9	226 ± 21	97	91
	5000 B	–	129 ± 12	277 ± 79	117	93
DMSO A		–	126 ± 19	284 ± 23		89
DMSO B		–	119 ± 11	288 ± 17		83
MMS	10	–	48 ± 5	57 ± 5	15	168
	15	–	138 ± 11	120 ± 18	26	230
	20	–	143 ± 40	99 ± 16	18	289[a]
DMSO A		+	97 ± 10	121 ± 19		160
DMSO B		+	95 ± 8	148 ± 36		128
NTO	250 A	+	87 ± 19	182 ± 41	89	96
	250 B	+	94 ± 47	193 ± 70	102	97
	500 A	+	96 ± 106	200 ± 45	81	96
	500 B	+	110 ± 23	192 ± 78	111	115
	1000 A	+	112 ± 18	219 ± 62	128	102
	1000 B	+	136 ± 23	198 ± 36	126	137
	2500 A	+	122 ± 23	183 ± 40	86	133
	2500 B	+	121 ± 21	185 ± 23	103	131
	5000 A	+	161 ± 29	249 ± 4	29	129
	5000 B	+	142 ± 22	214 ± 3	33	133
Acetone A		+	120 ± 12	197 ± 23		122
Acetone B			125 ± 12	182 ± 21		137
DMBA	1.0	+	146 ± 5	181 ± 31	85	161
	2.5	+	128 ± 84	133 ± 70	40	192
	5.0	+	175 ± 16	80 ± 52	7	438[a]

DMSO: dimethyl sulfoxide; MMS: methyl methanesulfonate; DMBA: 7,12-dimethylbenz(α)anthracene.
[a] Three times higher than the average mutant frequency of the corresponding solvent controls.

Table 4
Percent reticulocytes and micronucleated cells of rat peripheral blood dosed with NTO orally for 14 days.

Experiment (mg/kg-day)	Reticulocytes (%)		Micronucleated reticulocytes (%)	
	Female	Male	Female	Male
Untreated control	1.26 ± 0.135	2.29 ± 0.190	0.15 ± 0.015	0.15 ± 0.010
PEG control	1.26 ± 0.080	2.60 ± 0.297	0.14 ± 0.018	0.24 ± 0.033
1000	1.41 ± 0.318	2.37 ± 0.191	0.20 ± 0.029	0.27 ± 0.025
1500	0.96 ± 0.131	2.88 ± 0.481	0.23 ± 0.015	0.25 ± 0.049
2000	1.17 ± 0.138	2.39 ± 0.257	0.19 ± 0.030	0.21 ± 0.019
Positive control(200 mg/kg EMS)	0.78 ± 0.037	2.10 ± 0.719	0.32 ± 0.066*	0.30 ± 0.053*

* Significantly greater than the corresponding solvent control ($p = 0.005$ for females; $p = 0.047$ for male).

was performed with or without activation at concentrations from 0.1 to 5000 μg/ml. Based on the cytotoxicity data from the range-finding test, L5178Y TK$^{+/-}$ mouse lymphoma cells were treated with the test article at 10, 50, 100, 250, 500, 1000, 2500 and 5000 μg/ml in the mutation assay, with or without activation, for 4 h. After a 44 h expression period in the mutation assays, cultures were selected for cloning based on their cytotoxicity. Since the test article was nontoxic, the cultures treated with the five highest concentrations were selected for cloning. The plates were counted after 12 days incubation. The results showed all of the cultures that were treated with up to 5 mg/ml NTO either with or without activation had mutant frequencies that were similar to the average frequencies of concurrent solvent control cultures, which indicated a negative result. The results of the mouse lymphoma test are presented in Table 3.

The negative result was confirmed by a 24 h treatment assay without activation (data not shown).

Colony size distributions were performed for the solvent controls and positive controls and were found to be acceptable, i.e., the positive controls had bimodal distributions of large and small colonies.

3.4. Rat peripheral blood micronucleus assay

The results of the rat blood micronucleus assay are presented in Table 4. The frequency of micronucleated reticulocytes (%MN-RET) ranged from 0.20 to 0.23% in female rats and 0.21 to 0.27% in male rats treated with 1, 1.5, and 2 g/kg per day of NTO in neat PEG 200. Treatment with NTO did not statistically significantly ($p = 0.096$ and $p = 0.616$, respectively) increase the frequency of micronucleated reticulocytes (%MN-RET) in the peripheral blood of female or male rats. The positive control significantly increased, relative to the untreated control, the frequency of micronucleated reticulocytes in the peripheral blood of female and male rats ($p = 0.005$ and $p = 0.047$, respectively). These results indicate that NTO is not genotoxic in rat peripheral blood under the test conditions.

4. Discussion

The U.S. Army is developing insensitive munitions for future weapon systems under the direction of a Department of Defense (DOD) – wide initiative to improve the safety of munitions [27]. NTO is an energetic explosive candidate to replace highly sensitive munitions containing RDX and TNT [4,5]. Evaluation of chemical and physical properties of NTO showed it is less sensitive when compared to others munitions [28]. It also showed low mammalian toxicity when compared to RDX and TNT [29]. The acute oral LD 50 value of NTO was >5 g/kg in rats and mice [8]. We evaluated NTO for genotoxicity potentials in accordance with the USEPA's three-tiered system for the assessment of human health risks [30–32]. Our results showed that NTO was negative in three *in vitro* systems (Ames *Salmonella*, mouse lymphoma, and CHO chromosomal aberrations tests) and in the *in vivo* rat peripheral blood micronucleus assay.

NTO dissolved in water had a very low pH. Therefore, we conducted the *in vitro* assays by dissolving NTO in DMSO followed by dilution with water. For the *in vivo* rat study, it was suspended in neat PEG 200 to avoid low pH dosing solutions. In the bacterial range finding test with *salmonella* TA100 and *E. coli WP2uvrA*, it showed toxicity at 500 μg/plate and 1000 μg/plate, respectively, without activation, but it showed less toxicity in activated systems where we used higher concentrations (up to 5 mg/plate) for testing. This may be due to the difference in fate of NTO in the bacterial system with and without activation. The metabolism of NTO in *Bacillus licheniformis* preceded through an oxygen-insensitive nitro reduction leading to the primary amine 5-amino-1,2,4,-triazol-3-one followed by cleavage of the triazolone ring [11]. This difference in cytotoxicity with and without metabolic activation was not observed with mammalian CHO cells and mouse lymphoma cells. Nitroaromatic compounds such as TNT and tetryl are mutagenic in bacterial and mammalian systems [29,33]. Our results show NTO is negative in three *in vitro* and one *in vivo* system. Similarly the close analog of NTO, amitrole (3-aminotriazole) is also not mutagenic in *S. typhimurium* and *E. coli*/mammalian microsomal assay with or without activation, mouse lymphoma cells with activation and in Drosophila sex-linked recessive lethal assay [34,35].

NTO at doses up to 2 g/kg/day for 14 days, oral administration, did not induce significant increases in the frequency of micronucleated (MN) reticulocytes, indicating that it is not genotoxic in rats. The *in vivo* rodent micronucleus assay is recommended by the US EPA as one tier in evaluating genotoxic potentials of chemicals [30–32]. Historically, *in vivo* rodent micronucleus assays were conducted by evaluating the frequency of micronucleated erythrocytes in bone marrow or peripheral blood slide preparations. Flow cytometer based methods have recently been used in measuring micronuclei frequencies in erythrocytes and have been reported to be a sensitive system for the analysis of micronuclei [25,36–38]. The comparison of flow cytometer and microscopy based methods showed no significant differences in results obtained by the two methods with known genotoxic and non-genotoxic chemicals [25,39]. Our *in vivo* study indicated that NTO is not genotoxic in the peripheral blood of rats orally dosed with up to 2 g/kg/day of NTO for 14 days. *In vivo* studies, including toxicokinetic investigations to learn more about its fate in mammalian systems, and *in vivo* genotoxicity tests, such as the rodent dominant lethal assay, to investigate further any effects on gonads, may be considered for further studies.

Conflict of interest

No conflict of interest.

Acknowledgements

This work was funded by the U.S. Army Environmental Quality Technology (EQT) Ordnance Environmental Program (OEP) of

the U.S. Army Research, Development and Engineering Command (RDECOM), Environmental Acquisition and Logistics Sustainment Program. We would like to thank Erick Hangeland and Chandrark Patel for their continued interest and support of this effort.

References

[1] V.W. Manchot, R. Noll, Justus liebigs Annalen der Chemie 343 (1905) 1–27.
[2] K.Y. Lee, M.D. Coburn, 3-Nitro-1,2,4-triazol-5-one, a less sensitive explosive (LA-10302-MS), Los Alamos National Laboratory, Los Alamos, NM, 1985.
[3] M.J. Stosz, S.I. Collignon, C. Gotzmer, J.F. Leahy, Development of an insensitive explosive containing NTO, in: 38th Annual Bomb and Warhead Technical Meeting, Bomb and Armament Section, Air Armamment Division, ADPA, Albuquerque, N.M., Sandia National Laborarory, 1988 (cited in R.J. Spear, C.N. Louey, M. G. Wolfson. 1989. A preliminary assessment of NTO as an insensitive high explosive. MRL Technical Report, MRL-TR-89-18, Material Research Laboratory, Maribyrnong, Vic. Australia).
[4] R.J. Spear, C.N. Louey, M.G. Wolfson, A Preliminary Assessment of NTO as an Insensitive High Explosive, MRL Technical Report, MRL-TR-89-18, Material Research Laboratory, Maribyrnong, Vic., Australia, 1989.
[5] M.W. Smith, M.D. Cliff, NTO-based Explosive Formulations: A Technology Review. DSTO-TR-0796. Technical Report, Aeronautical and Maritime Research Laboratory, Melbourne, Vic., Australia, 1999.
[6] M. Beyard, U.S. Insensitive Munitions Policy Update, Presented at the 2007 NDIA IM-EM Meeting, 2007.
[7] NATO (North Atlantic Treaty Organization), Energetic Material Compendium, Version 4.0.1, NATO-Munitions Safety Information Analysis Center, Brussels, Belgium, 2010.
[8] J.O. London, D.M. Smith, A Toxicological Study of NTO. Technical Report No. LA-10533, Los Alamos National Laboratory, Los Alamos, NM, 1985.
[9] L.C.B. Crouse, E.M. LaFiandra, J.H. Houpt, A.J. O'Neill, M.R. Way, T.L. Hanna, M.J. Quinn, Toxicology study no. 85-XC-0A6W-08. Subchronic oral toxicity of 3-nitro-1,2,4-triazol-5-one (NTO) in rats. Technical Report, February 19, 2010. U.S. Army Public Health Command (Provisional), Aberdeen Proving ground, MD, 2010.
[10] L. LeCampion, M. DeLaForge, J.P. Noel, J. Ouazzani, Metabolism of ^{14}C-labelled 5-nitro-1,2,4-triazol-3-one by rat liver microsomes: evidence for participation of cytochrome P-450, Eur. J. Biochem. 248 (1997) 401–406.
[11] L. LeCampion, M. DeLaForge, J.P. Noel, J. Ouazzani, Metabolism of 14C-labelled 5-nitro-1,2,4-triazol-3-one (NTO): comparison between rat liver microsomes and bacterial metabolic pathways, J. Mol. Catal. B: Enzym. 5 (1998) 395–402.
[12] M.V. Haley, R.G. Kuperman, R.T. Checkai, Aquatic Toxicity of 3-Nitro-1,2,4-triazol-5-one (NTO), Edgewood Chemical Biological Center, U.S. Army Research, Development and Engineering Command, Aberdeen Proving Ground, MD, 2009, Technical Report No. ECBC-TR-726.
[13] OECD (Organization of Economic Cooperation and Development), Guideline for the testing of chemicals No. 471/472, Bacterial reverse mutation test; No. 473, *In vitro* mammalian chromosome aberration test; No. 474, Mammalian erythrocyte micronucleus test; No. 476, *In vitro* mammalian cell gene mutation test, Adopted July 21, 1997.
[14] B.N. Ames, J. McCann, E. Yamasaki, Methods for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test, Mutat. Res. 31 (1975) 347–436.
[15] D.M. Maron, B.N. Ames, Revised methods for the *Salmonella* mutagenicity test, Mutat. Res. 113 (1983) 173–215.
[16] M.H.L. Green, W.J. Muriel, Mutagen testing using trp+ reversion in *Escherichia coli*, in: B.J. Kilbey, al. et (Eds.), Handbook of Mutagenicity Test Procedures, Elsevier North Holland Biomedical Press, Amsterdam, 1977, pp. 65–94.
[17] G. Reddy, J. Song, M.S. Mecchi, M.S. Johnson, Genotoxicity assessment of two hypergolic energetic propellant compounds, Mutat. Res. 700 (2010) 26–31.
[18] H.J. Evans, Cytological methods for detecting chemical mutagens, in: A. Hollaender (Ed.), Chemical Mutagens: Principles and Methods for their Detection, vol. 4, Plenum Press, New York and London, 1976, pp. 1–29.
[19] S.M. Galloway, A.D. Bloom, M. Resnick, B.H. Margolin, F. Nakamura, P. Archer, E. Zeiger, Development of a standard protocol for i*n vitro* cytogenetic testing with Chinese hamster ovary cells: comparison of results for 22 compounds in two laboratories, Environ. Mutagen. 7 (1985) 1–51.
[20] S.M. Galloway, M.J. Armstrong, C. Reuben, S. Colman, B. Brown, C. Cannon, A.D. Bloom, F. Nakamura, M. Ahmed, S. Duk, J. Rimpo, G.H. Margolin, M.A. Resnick, G. Anderson, E. Zeiger, Chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells: evaluation of 108 chemicals, Environ. Mol. Mutagen 10 (Suppl. 10) (1987) 1–35.
[21] D. Clive, J. Spector, Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y$^{+/-}$ mouse lymphoma cells, Mutat. Res. 31 (1975) 17–29.
[22] D. Clive, K.O. Johnson, J. Spector, A.G. Batson, M. Brown, Validation and characterization of the L5178Y/TK$^{\pm}$ mouse lymphoma mutagen assay system, Mutat. Res. 59 (1979) 61–108.
[23] D. Clive, W. Caspary, P.E. Kirby, R. Krehl, M. Moore, J. Mayo, T.J. Oberly, Guide for performing the mouse lymphoma assay for mammalian cell mutagenicity, Mutat. Res. 189 (1987) 143–156.
[24] P. Kirby, Mouse lymphoma cell assays, in: J.F. Douglas (Ed.), Carcinogenesis and Mutagenesis Testing, Humana Press, Clifton, NJ, 1984, pp. 207–226.
[25] K.L. Witt, E. Livanos, G.E. Kissling, D.K. Torous, W. Caspary, R.R. Tice, L. Recio, Comparison of flow cytometry- and microscopy-based methods for measuring micronucleated reticulocyte frequencies in rodents treated with nongenotoxic and genotoxic chemicals, Mutat. Res. 649 (2008) 101–113.
[26] Litron Laboratories, MicroFlow Micronucleus Analysis Kit Instruction Manual, Litron Laboratories, Rochester, New York, 2003.
[27] K. Duncan, Insensitive munitions and the army: improving safety and survivability. Army Logist. PB700-02-1 34, 2002, 16–17.
[28] A. Becuwe, A. Delclos, Low-sensitivity explosive compounds for low vulnerability warheads, Propell. Expl. Pyrotech. 18 (1993) 1–10.
[29] J.V. Dilley, C.A. Tyson, G.W. Newell, Mammalian toxicological evaluation of TNT wastewaters: acute and subacute mammalian toxicity of TNT and LAP mixtures. vol. II. Final report. Contract No. DAMD 17-76-C-6050 AD-A080957. Menlo Park, CA; SRI International, 1978.
[30] K.L. Dearfield, A.E. Auletta, M.C. Cimino, M.M. Moore, Considerations in the US Environmental Protection Agency testing approach for mutagenicity, Mutat. Res. 258 (1991) 259–283.
[31] K.L. Dearfield, M.C. Cimino, N.C. McCarroll, I. Mauer, L.R. Valcovic, Genotoxicity risk assessment: a proposed classification strategy, Mutat. Res. 521 (2002) 121–135.
[32] M. Cimino, Comparative overview of current international strategies and guidelines for genetic toxicology testing for regulatory purposes, Environ. Mol. Mutagen. 47 (2006) 362–390.
[33] D.B. McGregor, C.G. Riach, R.M. Hastwell, J.C. Dacre, Genotoxic activity in microorganisms of tetryl,1,3-dinitrobenzene and 1,3,5-trinitrobenzene, Environ. Mutagen. 2 (1980) 531–541.
[34] E.L. Kier, D.J. Bruisick, A.E. Auletta, E.S. Von Halle, N.M. Brown, V.F. Simmon, V. Dunkel, J. Mccann, K. Mortelmans, M. Prival, T.K. Rao, V. Ray, The *Salmonella typhimurium*/mammalian microsomal assay: a report of the U.S. Environmental Protection Agency Gene-Tox program, Mutat. Res. 168 (1986) 69–240.
[35] R.N. Hill, L.S. Erdreich, O.E. Payntr, P.A. Roberts, S.L. Rosenthal, C.F. Wilkinson, Thyroid follicular cell carcinogenesis, Fundam. Appl. Toxicol. 12 (1989) 629–697.
[36] S.D. Dertinger, M.E. Bishop, J.P. McNamee, M. Hayashi, T. Suzuki, N. Asano, M. Nakajima, J. Saito, M.M. Moore, D.K. Torous, J.T. MacGregor, Flow cytometric analysis of micronuclei in peripheral blood reticulocytes. I. Intra-and interlaboratory comparison with microscopic scoring, Toxicol. Sci. 94 (2006) 83–91.
[37] Z. Cammerer, A. Elhajouji, W. Suter, In vivo micronucleus test with flow cytometry after acute and chronic exposures of rats to chemicals, Mutat. Res. 626 (2007) 26–33.
[38] J.T. MacGregor, M.E. Bishop, J.P. McNamee, M. Hayashi, N. Asano, A. Wataka, M. Nakajima, J. Saito, A. Aidoo, M.M. Moore, S.D. Dertinger, Flow cytometric analysis of micronuclei in peripheral blood reticulocytes. II. An efficient method of monitoring chromosomal damage in rats, Toxicol. Sci. 94 (2006) 92–107.
[39] K.A. Criswell, G. Krishna, D. Zielinski, G.A. Urda, P. Juneau, S. Bulera, M.R. Bleavins, Validation of a flow cytometric acridine orange micronuclei methodology in rats, Mutat. Res. 528 (2003) 1–18.